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Glycoprotein C of Herpes Simplex Virus Type 1 Plays a Principal Role in the Adsorption of Virus to Cells and in Infectivity

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The purpose of this study was to identify the herpes simplex virus glycoprotein(s) that mediates the adsorption of virions to cells. Because heparan sulfate moieties of cell surface proteoglycans serve as the receptors for herpes simplex virus adsorption, we tested whether any of the viral glycoproteins could bind to heparin-Sepharose in affinity chromatography experiments. Two glycoproteins, gB and gC, bound to heparin-Sepharose and could be eluted with soluble heparin. In order to determine whether virions devoid of gC or gB were impaired for adsorption, we quantitated the binding of wild-type and mutant virions to cells. We found that at equivalent input concentrations of purified virions, significantly fewer gC-negative virions bound to cells than did wild-type or gB-negative virions. In addition, the gC-negative virions that bound to cells showed a significant delay in penetration compared with wild-type virus. The impairments in adsorption and penetration of the gC-negative virions can account for their reduced PFU/particle ratios, which were found to be about 5 to 10% that of wild-type virions, depending on the host cell. Although gC is dispensable for replication of herpes simplex virus in cell culture, it clearly facilitates virion adsorption and enhances infectivity by about a factor of 10.

The entry of herpes simplex virus (HSV) into cells requires a cascade of events that occur at the cell surface and can be divided into two distinct phases: adsorption and penetration. Adsorption is mediated, at least in part, by the binding of virions to heparan sulfate moieties of cell surface proteoglycans (49). Penetration requires the activities of three virion glycoproteins (gB, gD, and gH) (2–4, 8, 12, 13, 16, 17, 26, 28, 38) and occurs by the fusion of the virion envelope with the cell plasma membrane (13, 20, 21, 41, 42, 48).

Heparan sulfate is a ubiquitous glycosaminoglycan found on proteoglycans in cell plasma membranes, in extracellular matrices, and in basement membranes. It is structurally and chemically similar to heparin, except that heparin is more highly sulfated (27). The conclusion that cell surface heparan sulfate serves as the receptor for HSV adsorption is based on several lines of evidence. First, HSV does not bind to cell surfaces stripped of heparan sulfate by enzymatic treatment with heparinase or heparitinase or to mutant cells that fail to synthesize heparan sulfate (40a, 49). The presence or absence of other glycosaminoglycans does not influence HSV adsorption. Experimental manipulations to reduce the levels of cell surface heparan sulfate lead to reduction in viral infection concomitant with the reduction in viral adsorption, indicating that the adsorption of virus to heparan sulfate is a required step in the pathway of infectious entry. Second, HSV virions can bind to immobilized heparin, and soluble heparin can block the binding of virions to cells (49), presumably by occupying heparan sulfate-binding sites on the virion surface. These results provided the molecular basis for the early observations that heparin could inhibit HSV infection (34, 44, 46). Third, heparin-binding proteins, such as platelet factor 4 (49) and fibroblast growth factor (FGF) (22), can inhibit HSV adsorption and plaque formation, probably by competing with virus for receptor sites associated with the cell surface heparan sulfate.

FGF is a generic name applied to a family of heparin-binding growth factors, most or all of which bind with high affinity to a family of protein receptors on the cell surface and with lower affinity to cell surface heparan sulfate (1). Recently, it was reported that an FGF receptor could provide a "portal of entry" into cells for HSV (22). This conclusion was based on results suggesting that radiolabeled HSV bound more efficiently to and was taken up in larger quantities by CHO cells that expressed a transfected FGF receptor than by control receptor-negative cells. Using these same cell lines, provided by C. Basilico (30), we have been unable to repeat the published results. Instead, we have found that HSV bound somewhat more efficiently to the receptor-negative cells than to the receptor-positive cells (which divide at a faster rate) and that there was no difference in the induction of early viral gene expression in the two cell lines (40b). Therefore, on the basis of information available at this time, the only cell surface components known to have any role in HSV adsorption are heparan sulfate glycosaminoglycans.

HSV specifies the synthesis of at least eight membrane glycoproteins (37), most or all of which are components of the virion envelope. Three of these glycoproteins (gB, gD, and gH) are clearly indispensable for viral replication, judged by the lethal effects of mutations in the genes encoding them (2, 3, 8, 26, 28, 38). It appears that at least two, and perhaps all three, of these glycoproteins are required for viral penetration and not for virion adsorption to cells. Virions devoid of gB or gD have been reported to bind to cells normally but to be defective for penetration (3, 26). In addition, antibodies specific for gB, gD, or gH can neutralize viral infectivity by blocking penetration without detectable effects on virion adsorption (12, 13, 16, 17). Five of the remaining HSV glycoproteins have been shown to be dispensable for viral replication under certain conditions,

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according to findings that mutants deleted for one or more of these glycoproteins can be propagated in cultured cells (15, 18, 29). Studies designed to detect sublethal defects in the infectivity of these mutants have not previously been reported.

Our approach to identifying the HSV glycoprotein(s) that mediates the binding of virus to cells was to determine which of the HSV glycoproteins had affinity for heparin and then to assess the adsorption capacity of mutant virions devoid of the heparin-binding glycoprotein(s). We found that both gB (an "indispensable" envelope glycoprotein required for penetration) and gC ("dispensable") bound to heparin-Sepharose and that virions devoid of gC exhibited significant impairment in adsorption and penetration. This impairment can account for the reduced PFU/particle ratio exhibited by the gC-negative mutant. Therefore, at least one of the dispensable glycoproteins has an important role in HSV infectivity.

These results obtained with HSV parallel results recently reported for the porcine herpesvirus pseudorabies virus (PRV) (32, 35, 36, 40, 47, 50). For both viruses, it appears that gC and the gC homolog of PRV (gIII) are dispensable for viral propagation in cell culture and yet play an important role in the adsorption of virus to cells. For gC-negative or gIII-negative mutants of these herpesviruses, an alternative, less-efficient means of adsorption, yet to be defined, must exist for infectious entry of virus into cells.

MATERIALS AND METHODS

Cells and viruses. HEp-2 cells and Vero cells were obtained from the American Type Culture Collection. The Vero-B24 cell line, which carries a functional gene for HSV type 1 (HSV-1) gB and was used for the propagation of a gB-negative HSV-1 mutant, was obtained by cotransfection of Vero cells with pRB2017 [a derivative of pBR322 containing gB gene in a *Bam*HI-*Sac*I fragment of HSV-1(F) DNA (provided by B. Roizman)] and pSV2-gpt. The transformed cells were selected for the ability to grow in the presence of mycophenolic acid and were shown to express HSV-1 gB after infection with HSV-2 or a gB-negative mutant (7a). The HEp-2 and Vero-B24 cells were passaged in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. The Vero cells were passaged in medium 199 supplemented with 5% fetal bovine serum. The virus strains used were HSV-1(KOS) (provided by P. Schaffer, Harvard Medical School), HSV-1(KOS)gC$^-$3 (provided by J. Glorioso, University of Pittsburgh), and HSV-1(KOS)K082 (provided by S. Person, University of Pittsburgh). The mutant gC$^-$3 expresses a truncated form of gC lacking the membrane-spanning domain and therefore produces virions devoid of gC (18). The mutant K082 contains a linker-inserted termination codon near the N terminus of the gB open reading frame and fails to produce gB (2). The wild-type strain KOS and the viable gC$^-$3 mutant were propagated in HEp-2 cells, whereas the defective K082 mutant was propagated in Vero-B24 cells. One passage of K082 through HEp-2 cells yields gB-negative virions.

Purification and quantification of virus. Virions purified as described by Cassai et al. (6) were used for all experiments. Briefly, roller bottles of HEp-2 cells were inoculated with virus at 3 to 5 PFU per cell for an adsorption period of 2 h. Following removal of the inoculum, cells were overlaid with medium 199 (in which the methionine concentration was reduced to 1/10 of normal levels for labeling with radioactive methionine) supplemented with 1% fetal bovine serum (199V) with or without [^{3}H]thymidine (20 μCi/ml), [^{35}S] methionine (15 μCi/ml), or [^{14}C]glucosamine (20 μCi/ml) and incubated at 37°C for 48 to 72 h. Virus was harvested and purified from infected-cell lysates and media by centrifugation through dextran gradients (Dextran T10; Pharmacia). Titers of infectious units were determined by plaque assays on both HEp-2 and Vero cells, and radioactivity was determined by liquid scintillation counting. The viral phenotype with respect to presence of gB or gC in virions was confirmed by performing Western blots (immunoblots) on all preparations. Just prior to use, virus was diluted 1:5 (vol/vol) in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$) and centrifuged at 25,000 rpm for 1 h in a Beckman 50 Ti rotor to concentrate the virus and remove most of the dextran. The virus pellet was suspended in an appropriate buffer. Infectivity titers and particle counts were performed on the resuspended virus.

Virions were quantitated either by electron microscopy or by comparing relative amounts of the VP5 (major capsid) protein. In the latter case, samples of each virion preparation were solubilized and the polypeptides were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Following silver staining (BioRad), the gels were scanned by optical densitometry. For quantification by electron microscopy, we adapted the methods originally described by Miller (33). Briefly, Formvar-coated, tabbed grids (Ted Pella, Inc.) were coated with poly-L-lysine and mounted in a Beckman EM 90 rotor. The wells of the rotor were then filled with samples of purified virus diluted in deionized, ultrafiltered water. Latex particles (Ted Pella) were diluted 1:25,000 and mixed with each sample as a reference. The virus and latex particles were pelleted onto the grids by centrifugation of the rotor at 20,000 lb/in^2 for 30 min in an airfuge. The grids were then fixed in 1% glutaraldehyde and negatively stained with 3% phosphotungstic acid. The grids were examined in a JEOL 100 CX-II electron microscope. Photographs of multiple fields within several windows of each grid were examined, and the particle numbers were calculated according to the equation $n = 250 V_f D/A$, where n equals the number of particles per milliliter, V_f is the average number of particles per field, A is the surface area (in square millimeters) of the field determined with reference to latex particle size, and D is the dilution factor of the particle suspension. The total area of the surface onto which the virions were pelleted was 25 mm^2, and each sectored well of the rotor held 0.1 ml.

Viral plaque assays. Viral plaque assays were performed in duplicate. HEp-2 or Vero cells in 25-cm^2 flasks were inoculated with virus in PBS. After 2 h of adsorption, the viral inoculum was removed and the cells were overlaid with medium 199 supplemented with 1% fetal bovine serum and 0.1% pooled human gamma globulin (199O). Plaques were counted after 3 days of incubation. Vero cells were fixed and stained with Giemsa for the visualization of plaques. In order to visualize the plaques on HEp-2 cells, an immunoassay adapted from a previously published procedure (19) was used. Briefly, the medium was aspirated and the cells were washed with PBS supplemented with 3% bovine serum albumin. The cells were incubated sequentially with an anti-gB monoclonal antibody (II-105) diluted 1:1,000, biotinylated goat anti-mouse immunoglobulin G (BRL), and streptavidin–β-galactosidase (BRL). Each incubation was for 30 min at room temperature, with washing after removal of the reagent. Finally, bluogal (BRL) substrate was added

and the monolayers were overlaid in 0.7% agarose. Blue plaques were visualized and counted.

Heparin affinity chromatography. Purified radiolabeled virus (1 ml containing 6×10^8 to 10×10^8 PFU of [^{35}S] methionine-labeled or [^{14}C]glucosamine-labeled KOS) was diluted fivefold in PBS and centrifuged at 25,000 rpm in a Beckman 50 Ti rotor for 2 h. The virus pellet was lysed on ice in 200 μl of PBS containing 0.5% Nonidet P-40 and 0.5% sodium deoxycholate (PBS-NP40-DOC) and then centrifuged at 25,000 rpm for 1 h in a Beckman 50 Ti rotor to remove nucleocapsids. A sample (180 μl) of the supernatant containing viral glycoproteins was applied to a 2-ml column of heparin-Sepharose that had been equilibrated with PBS-NP40-DOC. The column was washed with 10 volumes of PBS-NP40-DOC. Bound glycoproteins were eluted with PBS-NP40-DOC containing heparin at 2 mg/ml. Fractions containing material that passed straight through the column and the heparin-eluted material were separately pooled and then concentrated, using Centricon-30 concentrators, to volumes equivalent to that of the applied material. The material was analyzed by immunoprecipitation and SDS-PAGE (15).

Extracts of HSV glycoproteins were also mixed with heparin-Sepharose in tubes to assess the affinity of the glycoproteins for heparin. Extracts were prepared from [^{35}S]methionine-labeled virions of gC$^-$3 or K082 as described above. Samples (100 μl) were mixed with 100 μl of a 50% suspension of heparin-Sepharose and placed on ice for 1 h with occasional agitation. The heparin-Sepharose beads with attached proteins were pelleted (the supernatant was recovered for analysis of unbound proteins), washed once with 500 μl of PBS-NP40-DOC, and resuspended in 150 μl of PBS-NP40-DOC containing heparin at 5 mg/ml. After an additional 1 h on ice, the beads were again pelleted and the heparin-eluted proteins were recovered in the supernatant fraction. Samples of the original extract, the unbound proteins, and the heparin-eluted proteins were analyzed by SDS-PAGE.

Immunoprecipitation and Western blots. Glycoprotein lysate (100 μl) was incubated for 30 min on ice with 2 μl of the appropriate ascites fluid, and then 100 μl of 10% Formalin-fixed *Staphylococcus aureus* was added. After 30 min on ice, the mixture was spun for 2 min in an Eppendorf centrifuge and then washed as follows: twice with PBS-NP40-DOC, once with high-salt PBS (500 mM NaCl)-NP40-DOC, and once with deionized water. The pellet was suspended in 50 μl of SDS sample buffer (10 mM sodium phosphate [pH 7.0], 1% SDS, 0.1 M dithiothreitol, 10% glycerol, 0.001% bromphenol blue), boiled for 2 min, and then loaded onto an 8.5% SDS-polyacrylamide slab gel (*N-N'*-diallyltartar diamide cross-linked). Following SDS-PAGE, the gel was fixed in 7% acetic acid, soaked in Amplify (Amersham), vacuum dried, and exposed to autoradiographic film at −70°C.

For preparation of immunoblots, after SDS-PAGE proteins were transferred to nitrocellulose (Schleicher & Schuell) by Western transfer in 10 mM Tris–150 mM glycine–20% methanol (45). The nitrocellulose was incubated for 1 h at 25°C in PBS containing 5% skim milk and then in PBS–5% milk containing the appropriate antiserum (1:1,000 to 1:2,000 dilution) at 4°C overnight. Following several washes in PBS containing 0.3% Tween 20, the nitrocellulose blot was incubated for 1 h with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G diluted 1:2,000 in PBS containing 5% bovine serum albumin. The blot was rinsed several times with PBS–0.3% Tween 20, once with PBS, and once with 100 mM Tris HCl (pH 8.8)–1 mM $MgCl_2$ (rinse buffer). The blot was then immersed in the rinse buffer supplemented with 5-bromo-4-chloro-3-indoyl phosphate (0.05 mg/ml) and Nitroblue Tetrazolium (0.1 mg/ml) until the desired color development was achieved.

Binding of radiolabeled virus to cells. Confluent monolayers of HEp-2 or Vero cells in glass scintillation vials were pretreated for 1 h at 37°C with PBS containing 1% bovine serum albumin (PBS-BSA) in order to block nonspecific virus adsorption. The cells were then inoculated with serial twofold dilutions of radiolabeled, purified virions in PBS-BSA and placed on a shaker at 4°C for 5 h. After the adsorption period, the virus inoculum was removed and the cells were washed three times in PBS. Ecolume (ICN BioMedicals) was added to the scintillation vials, and the radioactivity was counted in a scintillation counter. The infectivity titers and viral particle concentrations of the preparations utilized in the binding studies were determined.

In one experiment, an alternative procedure was used for the binding assay. Various concentrations of KOS, gC$^-$3 and K082 virions were adsorbed to HEp-2 cells in 96-well (round-bottom) plates for 2 h at 4°C. Following three rinses with PBS, the cells were solubilized in PBS containing 1% SDS and 1% Triton X-100 and transferred to vials for liquid scintillation counting. Relative particle numbers were determined by quantitating the relative amounts of VP5 in each preparation as described above.

Assay for rate of virus penetration. The rate of virus penetration was assessed by determining the rate at which adsorbed virus became resistant to inactivation by a low-pH citrate buffer. The assessment procedure was adapted from methods described by Huang and Wagner (20) and Highlander et al. (17). Confluent HEp-2 or Vero cells in 25-cm^2 flasks were inoculated with purified wild-type or gC-negative virus (1,000 PFU per well) for an adsorption period of 2 h at 4°C. The inoculum was removed, and the cells were washed three times in complete PBS. The cells were then overlaid with 199V and shifted to 37°C to allow viral penetration to proceed. At selected times after temperature shift, experimental wells were treated with 1 ml of citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl [pH 3.0]) and control wells were treated with 1 ml of complete PBS for 1 min. The monolayers were then washed three times with PBS and overlaid with 199O. Plaques were visualized and counted as described above. Experiments were performed in triplicate.

Heparin inhibition of viral infection. Cultures of HEp-2 and Vero cells were inoculated with purified KOS or gC$^-$3 virions in the presence or absence of increasing concentrations of heparin (Sigma). After 2 h of adsorption at 37°C, the cells were washed three times in PBS and overlaid with 199O. Plaques were counted as described above. Experiments were performed in duplicate at two dilutions of virus input.

Statistical analysis. Data are presented with standard deviations. Student unpaired, two-tailed *t* tests were performed.

RESULTS

Identification of HSV glycoproteins with affinity for heparin-Sepharose. Envelope proteins were extracted from wild-type KOS virions with PBS-NP40-DOC and applied to heparin-Sepharose columns. The columns were washed thoroughly to remove unbound proteins, and the bound proteins were eluted with buffer containing heparin at 2 mg/ml. After separately concentrating the material that flowed straight through the columns and the material that

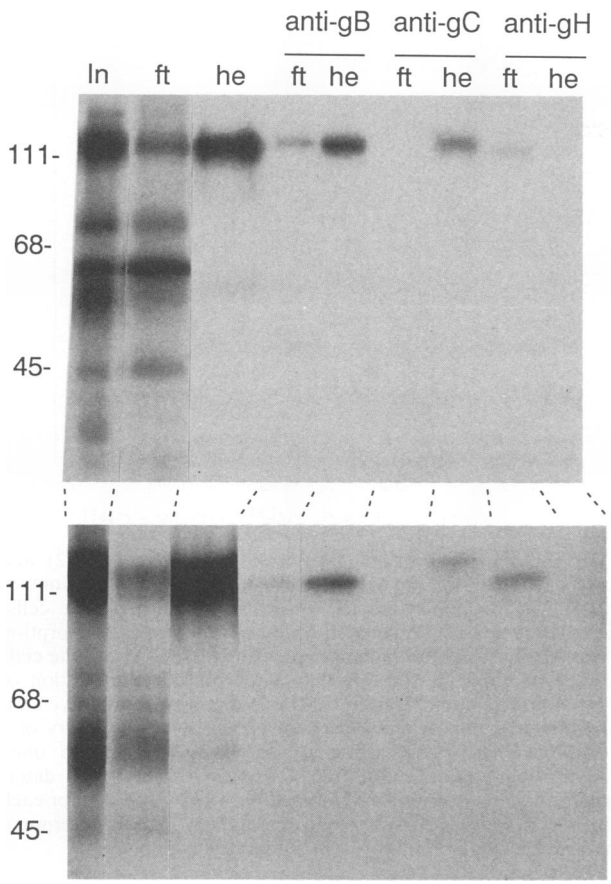

FIG. 1. Affinity for heparin-Sepharose of envelope glycoproteins extracted from KOS virions. The virions were radiolabeled with [^{35}S]methionine (upper panel) or [^{14}C]glucosamine (lower panel). The envelope proteins were extracted with PBS-NP40-DOC, and the solubilized material was applied to heparin-Sepharose columns. Fractions containing material that did not bind to the column (flowthrough [ft]) were pooled, as were the fractions containing material that bound to the column and could be eluted with heparin at 2 mg/ml (heparin eluted [he]). The two pooled sets of fractions were concentrated (to volumes equivalent to that of the starting material [In]) and analyzed by SDS-PAGE directly or after immunoprecipitation with monoclonal antibodies specific for gB, gC, or gH. The numbers on the left indicate the positions and molecular sizes (in kilodaltons) of size markers.

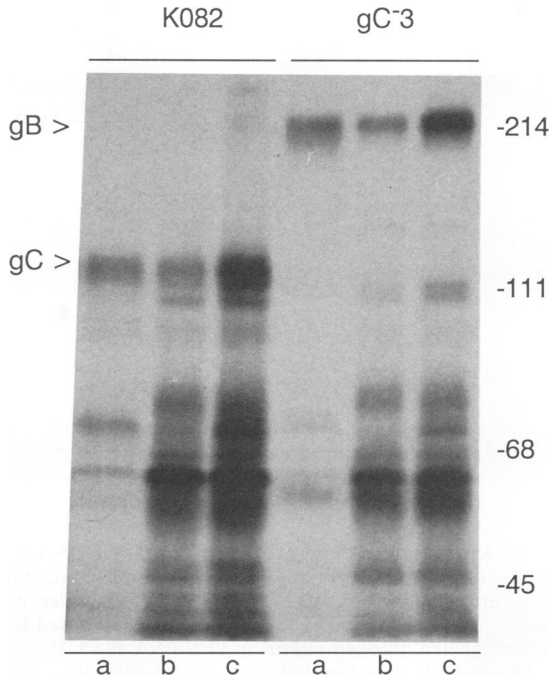

FIG. 2. Affinity for heparin-Sepharose of envelope glycoproteins extracted from gC$^-$3 or K082 virions. The virions were labeled with [^{35}S]methionine. The envelope proteins were extracted with PBS-NP40-DOC, and the solubilized material was mixed with heparin-Sepharose beads in tubes on ice for 1 h with occasional mixing. The beads were pelleted, and the supernatant fractions were recovered as unbound material. After the beads were washed, heparin (5 mg/ml) was added to elute the bound proteins. Equivalent samples of the input material (lanes c), unbound fraction (lanes b), and heparin-eluted fraction (lanes a) were analyzed by SDS-PAGE. Because the samples were not boiled prior to electrophoresis, gB migrated as an oligomer (7, 39) instead of as a monomer and was well resolved from gC. Molecular size markers are as described in the legend to Fig. 1.

was eluted with heparin, equivalent samples of the extracts applied to the columns, the flowthrough fractions, and the heparin-eluted fractions were analyzed by SDS-PAGE. The results of two experiments done with KOS virions, labeled either with [^{35}S]methionine or with [^{14}C]glucosamine, are shown in Fig. 1. Almost all the labeled material that bound to the heparin-Sepharose and could be eluted with heparin migrated on the gel with an apparent monomer molecular size range of 100 to 130 kDa, the size range characteristic of three of the HSV-1 glycoproteins, gB, gC, and gH. Unheated samples were also run on the gels (lanes not shown), revealing that gB oligomers (7, 39) were present in the heparin-eluted fractions but not in the flowthrough fractions. Samples of the flowthrough and heparin-eluted fractions were mixed with monoclonal antibodies to obtain immunoprecipitates of the individual glycoproteins. Analysis of the immunoprecipitates by SDS-PAGE revealed that the heparin-eluted fractions contained most of the gB and gC, whereas the flowthrough fractions contained most of the gH (Fig. 1).

To determine whether the binding of gC to heparin-Sepharose depended on the presence of gB and vice versa, extracts were prepared from purified, [^{35}S]methionine-labeled mutant virions that were devoid either of gC (gC$^-$3) or of gB (K082). The two extracts were separately mixed with samples of heparin-Sepharose in tubes. After time was allowed for binding, the unbound proteins were removed, the Sepharose beads were washed, and the bound proteins were eluted with heparin at 5 mg/ml. SDS-PAGE analysis of the input material, the unbound fractions, and the bound, heparin-eluted fractions revealed that gB and gC could each bind to heparin-Sepharose in the absence of the other (Fig. 2). In this batchwise affinity experiment, the efficiency of binding of the heparin-binding glycoproteins was lower and the background was higher than in the chromatography experiments (Fig. 1), probably because of less favorable ratios of extract and heparin-Sepharose and less favorable washing conditions. These experiments, or any others that could be done with unfractionated extracts of envelope proteins, do not rule out the possibility that the affinity of gC or gB for heparin-Sepharose could be influenced by other components of the extract. This possibility should be ad-

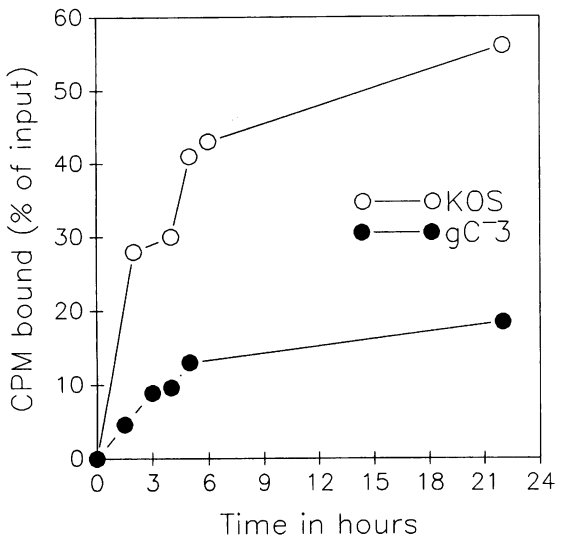

FIG. 3. Kinetics of adsorption to HEp-2 cells of KOS and gC⁻3 virions. Confluent monolayers of HEp-2 cells in glass scintillation vials were pretreated for 1 h at 37°C with PBS-BSA in order to block nonspecific virus adsorption. The cells were then exposed to [^{35}S] methionine-labeled, purified virions in PBS-BSA at 4°C for various times. After the adsorption period, the virus inoculum was removed, the cells were washed three times with PBS, and the cell-bound radioactivity was quantitated. Each point represents the average value from duplicate samples. The error bars indicating standard deviations are not seen because they are shorter than the radii of the symbols.

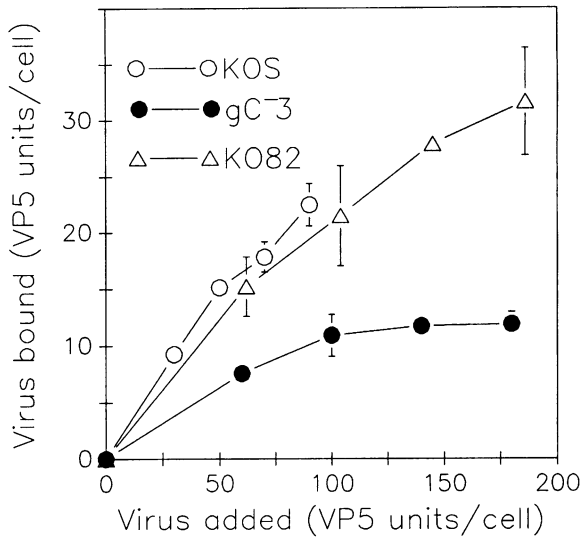

FIG. 4. Adsorption of mutant virions (gC⁻3 and K082) and wild-type KOS virions to HEp-2 cells. Various concentrations of purified, [^{35}S]methionine-labeled virions were added to the cells, which had been plated in 96-well round-bottom plates. Adsorption was for 2 h at 4°C. After unbound virus was washed away, the cells were solubilized and transferred to vials for the quantitation of cell-bound radioactivity by scintillation counting. The relative concentrations of input virions were determined by densitometry of a silver-stained SDS-PAGE gel and are expressed as VP5 units (relative rather than absolute units). The bound virus was determined from the known ratios of radioactivity and VP5 units for each virus preparation. Each point is the average of triplicate determinations, and the error bars represent standard deviations.

dressed in the future with purified forms of the glycoproteins.

Adsorption of mutant and wild-type virions to cells. To quantitate the adsorption of virus to cells without the complication of viral entry and possible turnover of cell receptors for virus, the binding studies were done at 4°C. Pilot experiments were performed to determine the time required for viral binding to reach equilibrium. HEp-2 cells plated on the bottoms of glass scintillation vials were exposed to purified radiolabeled KOS or gC⁻3 virus at 4°C for different times. At the times indicated, the cells in duplicate vials were washed thoroughly and then scintillation fluid was added for quantitation of the bound radioactivity. As shown in Fig. 3, 5 to 6 h is required for the binding reaction to approach equilibrium for both strains of virus.

Figures 4 and 5 present the results of two experiments in which the ability of mutant virions to bind to HEp-2 cells was compared with that of wild-type virions. In these experiments, two different methods were used to determine the concentrations of virions added to the cells for the binding reaction: either counting of virions by electron microscopy or quantitation of relative numbers of virions by comparisons of the amounts of VP5 (the major capsid protein) present in each preparation. The two methods yielded similar results. Figure 4 shows that, at equivalent concentrations of input virions (over the range tested), there was no significant difference between the wild-type parental strain (KOS) and the gB-negative mutant (K082) in the number of virions bound to HEp-2 cells, confirming previous findings (3). On the other hand, significantly fewer gC-negative virions (gC⁻3) than either wild-type or gB-negative virions bound to the HEp-2 cells (Fig. 4 and 5). The results presented in Fig. 5 were obtained at conditions approaching equilibrium (5 h of adsorption at 4°C). They show that the higher concentrations of gC⁻3 added were sufficient to saturate HEp-2 cell receptors at about 8,000 to 10,000 virions per cell, whereas at equivalent input concentrations of KOS virions, receptors for KOS virus were not saturated. Moreover, the number of KOS virions that could bind per HEp-2 cell must be in excess of 50,000. These results suggest that there could be fewer HEp-2 cell receptors for gC⁻3 virions than for KOS virions, implying that the receptors for the two viruses are different. On the other hand, a marked difference in affinity of a single class of receptors for the two viruses could also contribute to the differences in binding observed.

Specific infectivities of gC⁻3 and KOS virions and relative rates of penetration. Purified preparations of gC⁻3 and KOS virions were characterized with respect to virion concentration (by particle counts in the electron microscope) and titer of infectious units on HEp-2 cells and Vero cells. The results presented in Table 1 show that both viruses had lower specific infectivities on HEp-2 cells than on Vero cells. The most important point of Table 1, however, is that the PFU/particle ratio for gC⁻3 was much less than that of KOS on both cell types. Because the impairment in adsorption of gC⁻3 virions to HEp-2 cells is not sufficient to account fully for the reduced specific infectivity, it seems likely that the fraction of adsorbed gC⁻3 virions capable of initiating infection during a given time interval is less than the equivalent fraction of adsorbed wild-type KOS.

To test the possibility that penetration of adsorbed gC⁻3 virions was slower than that of adsorbed KOS virions, rates of penetration were assessed by using a protocol described previously by others (17, 20). Purified gC⁻3 or KOS virions

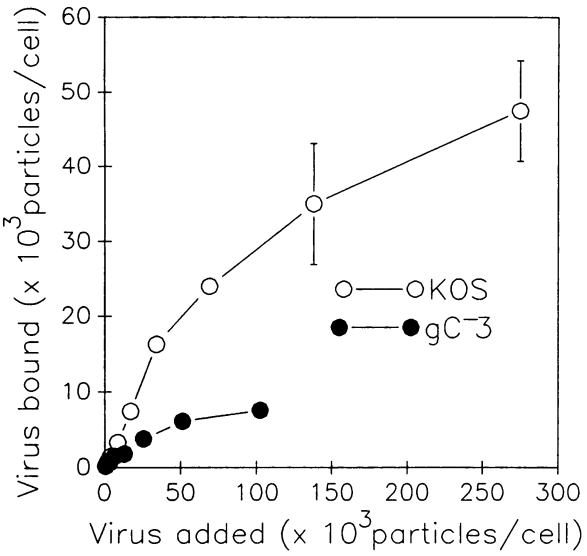

FIG. 5. Adsorption of mutant gC⁻3 virions and wild-type KOS virions to HEp-2 cells. Various concentrations of purified, [^{3}H] thymidine-labeled virions were added to HEp-2 cells, which had been plated on the bottoms of glass scintillation vials. Adsorption was for 5 h at 4°C. After unbound virus was washed away, scintillation fluid was added to the vials for the quantitation of cell-bound radioactivity. The concentrations of input virions were determined by particle counting in the electron microscope. The bound virus was determined from the known ratio of radioactivity and particle number in each virion preparation. Each point is the average of duplicate determinations, and the error bars represent standard deviations.

were adsorbed to HEp-2 cells or to Vero cells for 3 h at 4°C. After any unbound virus was washed away, the cells were incubated at 37°C for various times and then exposed to a low-pH citrate buffer (or to PBS as a control) to inactivate any bound virus that had not yet penetrated the cell. The results presented in Fig. 6 show that there was a significant delay in the penetration of adsorbed gC⁻3 virus compared with that of KOS virus but that the rates of penetration for the two viruses were then comparable.

Effect of heparin on the infectivity of gC⁻3 and KOS virions. To begin exploring the nature of the interaction between the gC-negative gC⁻3 virus and the cell surface, we tested whether heparin could block the infectivity and adsorption of this mutant. The results presented in Fig. 7 confirm our previous findings that the infectivity of KOS on HEp-2 cells is very sensitive to inhibition by heparin (49). These results also show that the infectivity of gC⁻3 on HEp-2 cells is equally sensitive to inhibition by heparin. Somewhat higher concentrations of heparin are required to achieve equivalent inhibition of infectivity on Vero cells, however, and gC⁻3 is considerably more resistant to heparin

TABLE 1. Specific infectivity (PFU/particle ratio) of wild-type and gC-negative virus on different cell types[a]

Cell line	10^{-2} PFU/particle	
	KOS	gC⁻3
Vero	4.30 ± 1.4 (100)	0.46 ± 0.09 (11)
HEp-2	2.15 ± 0.6 (50)	0.12 ± 0.04 (3)

[a] Plaque dishes of Vero or HEp-2 cells were inoculated with purified wild-type (KOS) or gC-negative (gC⁻3) virions for 2 h at 37°C. Dishes were overlaid with medium 199O, and plaques (PFU) were counted after 3 days of infection. Particle number for each preparation of purified virus was determined by electron microscopy (mean values obtained were 8.25×10^{10} particles per ml for KOS and 5.8×10^{10} particles per ml for gC⁻3). Specific infectivity was calculated in three separate experiments performed in duplicate at two different dilutions and is presented as mean ± standard error. The relative specific infectivity (percentage with KOS on Vero taken as 100%) is shown in parenthesis. The differences between KOS and gC⁻3 with respect to specific infectivities on both cell lines are significant ($P = 0.021$ on Vero cells and $P = 0.008$ on HEp-2 cells). The difference in the ability of gC⁻3 to infect Vero cells and HEp-2 cells is also highly significant ($P = 0.006$). However, the differences in specific infectivity of KOS on the two cell lines is not statistically significant ($P = 0.15$).

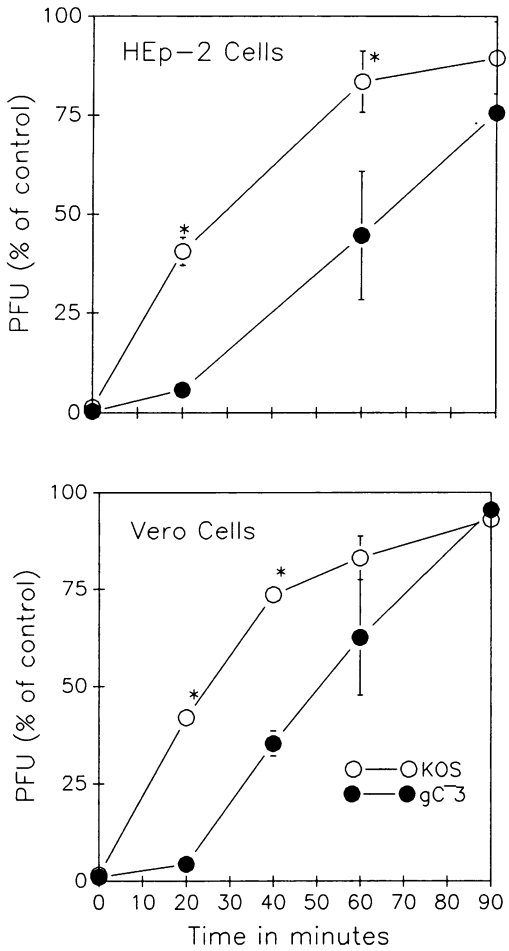

FIG. 6. Rates of penetration into HEp-2 cells and Vero cells for mutant gC⁻3 virions and wild-type KOS virions. Confluent monolayers of cells in 25-cm^2 flasks were inoculated with 1,000 PFU of purified virus for a 3-h adsorption period at 4°C. After removal of the inoculum and thorough washing of the cells, the flasks were shifted to 37°C to allow penetration to proceed. At selected times after the temperature shift, duplicate monolayers were treated for 1 min with 1 ml of citrate buffer or with PBS. The monolayers were then washed three times with PBS and overlaid with 199O. The plaques were counted after 3 days. The number of plaques on the control cultures treated with PBS were essentially the same for all time points, and the average value was taken as 100%. The results are presented as the PFU surviving citrate treatment at each time point expressed as a percentage of the PBS control value. Each point is the average of triplicate determinations, and the error bars represent standard deviations. The asterisks denote time points for which the differences between the two viruses are significant ($P < 0.01$; Student unpaired t test).

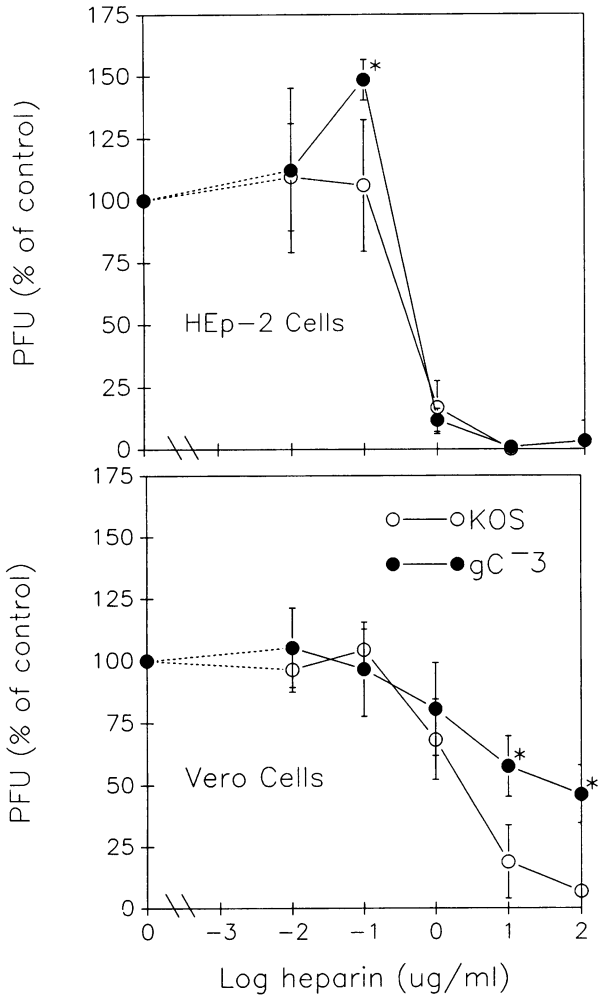

FIG. 7. Effects of heparin on plaque formation by gC$^-$3 and KOS on HEp-2 cells and Vero cells. Cultures of the cells in 25-cm^2 flasks were inoculated with purified virus in the absence or presence of various concentrations of heparin. After a 2-h adsorption period at 37°C, the cells were washed three times with PBS and overlaid with 199O. Plaques were counted after 3 days. The results are presented as PFU formed in the presence of heparin as a percentage of PFU detected in the absence of heparin. Five different experiments were performed in duplicate for each cell line. Each point represents the mean of values obtained for all five experiments, and the error bars represent standard deviations. The asterisks indicate points for which the differences between the two viruses are significant ($P < 0.01$).

inhibition than is KOS (on Vero cells). Similar experiments were done to assess the effects of heparin on the adsorption of radiolabeled gC$^-$3 and KOS to both HEp-2 cells and Vero cells, with results that resemble those shown in Fig. 7 (data not shown). We also showed that heparitinase treatment of HEp-2 cells reduced the amount of gC$^-$3 virus that could bind to the cells, just as heparitinase treatment reduced the binding of KOS virus (49). Finally, preliminary results indicate that gC$^-$3 fails to bind to mutant CHO cells devoid of heparan sulfate and fails to initiate the abortive replicative cycle that can be observed in wild-type, heparan sulfate-positive CHO cells (15a). All these results suggest the possibility that gC$^-$3, like KOS, requires the presence of cell surface heparan sulfate for the initial attachment of virus to cells, at least to HEp-2 cells and CHO cells. The requirements for adsorption may be different for Vero cells. It should also be noted that low doses of heparin (about 0.1 μg/ml) reproducibly enhanced the adsorption of gC$^-$3 to HEp-2 cells and the number of plaques observed (Fig. 7). We have as yet no explanation for this phenomenon.

DISCUSSION

It appears that gC is principally responsible for the adsorption of wild-type HSV virions to cells. This conclusion emerges from the finding that significantly fewer gC-negative virions than wild-type virions can bind to cells under conditions approaching saturation of available sites on the cell surface. Inasmuch as gC has heparin-binding activity, it seems likely that gC mediates the initial binding of virions to cell surface heparan sulfate.

As mentioned in the Introduction, PRV encodes a homolog of gC (gIII) which also significantly enhances the adsorption of virus to cells, even though it is not absolutely essential for virion infectivity (36, 40, 47, 50). In addition, it appears that PRV gIII (like HSV gC) mediates the adsorption of wild-type PRV to cell surface heparan sulfate inasmuch as heparin blocks the adsorption and infectivity of PRV, both virus and solubilized gIII have affinity for heparin-Sepharose, and treatment of cells with heparinase blocks adsorption and infectivity of PRV (32).

Although the initial interactions of wild-type HSV and PRV with cells appear to have features in common, gC-negative HSV and gIII-negative PRV exhibit some important differences in requirements for their diminished levels of infectivity. One difference is that gIII-negative PRV virions apparently bind very weakly to cells and can readily be removed by washing (40), whereas we found that although fewer gC-negative HSV virions than wild-type virions bound to cells, those that bound could not be removed by simple washing (unpublished observations). The gC-negative virions did, however, remain accessible to inactivation by acid much longer than did wild-type virions.

Another difference is that the infectivity of a gC-negative HSV mutant appears to require the presence of cell surface heparan sulfate, whereas the infectivity of a gIII-negative PRV mutant apparently does not (32). Specifically, heparin inhibited the adsorption and infectivity of HSV gC$^-$3 (to a much greater extent on HEp-2 cells than on Vero cells) but not those of a gIII-negative PRV mutant. Also, heparitinase (or heparinase) treatment of cells inhibited the adsorption and infectivity of HSV gC$^-$3 but not those of a gIII-negative PRV mutant. In fact, treatment of cells with low concentrations of heparinase actually enhanced the infectivity of the PRV mutant (32). This phenomenon may be related to the enhancement of infectivity (and binding) we have observed with low doses of heparin and also reported for HSV-2 gC-negative virions with low doses of polylysine (5).

Our results suggest that, unlike gIII-negative PRV, gC-negative HSV is still dependent on interactions with cell surface heparan sulfate for viral entry. There must be another viral glycoprotein that can mediate the interaction with heparan sulfate moieties. Judging from preliminary evidence, the most obvious possibility to explore is that gB, which is also a heparin-binding glycoprotein, can substitute for gC in mediating the adsorption of virus to cells. It is of interest that the PRV homolog of HSV gB (gII) does not bind strongly to heparin-Sepharose in the absence of gIII (32), whereas the binding of HSV gB to heparin-Sepharose is not

influenced by the presence or absence of gC (Fig. 2 and unpublished observations).

Although these considerations raise the possibility that gB could mediate the adsorption to cells of gC-negative HSV virions, gB appears not to make a large contribution to the adsorption of wild-type virus. This conclusion emerges from the fact that no evident impairment of adsorption has been observed for gB-negative virions (3). It is relevant to note that gB and gC are present in different spikes projecting from the virion envelope; gB forms the most prominent spikes, which extend about 14 nm from the virion envelope, and gC forms long slender structures which appear to extend about 20 nm from the envelope (43).

The alternative means of adsorption utilized by the gC-negative HSV virions is not only less efficient with respect to the number of virions that can bind to cells but is also less efficient with respect to penetration of the bound virions. This latter point is evident from our finding that the cell-bound gC-negative virions capable of forming plaques remained accessible to inactivation by acid longer than bound wild-type virions and also from the fact that the lower specific infectivity of gC-negative virions cannot be accounted for solely by their reduced abilities to bind to cells. The specific infectivity of the gC-negative virions was observed to be about 5 to 10% that of wild-type virions, depending on the cell type used for the infectivity assay, whereas the adsorption activity of the gC-negative virions on HEp-2 cells was about 20% that of wild-type virions.

Other recent studies have provided indirect evidence of a role for gC in HSV infectivity (5, 25). These studies followed up on an earlier observation that neomycin inhibited the formation of plaques on BHK cells by HSV-1 but not by HSV-2 (23). It was shown that expression of HSV-2 gC confers resistance to neomycin on BHK cells (but not on HEp-2 cells or Vero cells) and that gC-negative HSV or viruses expressing HSV-1 gC are sensitive to neomycin on all three cell types (5). It has been assumed that neomycin acts by inhibiting HSV adsorption, mostly because the agent must be present during adsorption to have its effect (the only experiment in which adsorption of virions was directly measured was done at 37°C [24] under conditions in which effects of penetration on adsorption could not be controlled). However, agents such as dithiothreitol, which inhibit HSV plaque formation, must be present during the adsorption period to block HSV plaque formation, yet have little or no effect on the amount of virus that adsorbs to the cells (42, 48). Therefore, the possibility exists that neomycin blocks some step subsequent to the adsorption mediated by gC and that HSV-2 gC somehow abrogates the inhibitory activity on certain cell types. A prediction based on this hypothesis is that neomycin might block the adsorption of gC-negative HSV but not of wild-type HSV.

Not only does gC have heparin-binding activity and mediate the adsorption of HSV to cells, but it also binds to the C3b fragment of the third component of complement (9, 10, 31). It has been shown that gC can accelerate the decay of the C3 convertase of the alternative pathway (11). This activity could account for the protection against complement-mediated damage to virus or infected cells observed when gC is present and not observed when it is absent (14, 31). Although these results obtained with in vitro assays suggest that gC could play a role in vivo in protecting virus or infected cells from complement-mediated damage, the physiological importance of the C3b-binding activity of gC is not yet known. The question arises as to the independence or interdependence of the heparin-binding and C3b-binding activities of gC. It will be important to determine in future studies whether C3b influences the infectivity of HSV.

Experiments published to date have addressed the role in adsorption of only three of the HSV glycoproteins (gB, gC, and gD). In light of the surprising result that a dispensable glycoprotein plays an important role in virion adsorption, future studies must also address the possibility that other dispensable or nondispensable glycoproteins influence HSV adsorption to cells. Our finding that heparin more effectively inhibits HSV infection of HEp-2 cells than of Vero cells (particularly for the gC-negative mutant) also suggests that HSV may have alternative means of binding to different cell types as well as alternative means of binding to a single cell type.

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